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Synthesizing the role of epigenetics in the response and adaptation of species to climate change in freshwater ecosystems

Running title: Epigenetics for adaptation in freshwaters

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Abstract

Freshwater ecosystems are amongst the most threatened ecosystems on Earth. Currently, climate change is one of the most important drivers of freshwater transformation and its effects include changes in the composition, biodiversity and functioning of freshwater ecosystems. Understanding the capacity of freshwater species to tolerate the environmental fluctuations induced by climate change is

critical to the development of effective conservation strategies. In the last few years, epigenetic mechanisms were increasingly put forward in this context because of their pivotal role in gene-environment interactions. In addition, the evolutionary role of epigenetically inherited phenotypes is a relatively recent but promising field. Here, we examine and synthesize the impacts of climate change on freshwater ecosystems, exploring the potential role of epigenetic mechanisms in both short- and long-term adaptation of species. Following this wrapping-up of current evidences, we particularly focused on bringing together the most promising future research avenues towards a better understanding of the effects of climate change on freshwater biodiversity, specifically highlighting potential molecular targets and the most suitable freshwater species for future epigenetic studies in this context.

Keywords: climate change; freshwater biodiversity; adaptation; epigenetic mechanisms; phenotype; transgenerational epigenetic inheritance.

Introduction

Worldwide, human demand for freshwater involves disruptive exploitation in numerous terrestrial and aquatic ecosystems (Corcoran, 2010; Jackson et al., 2001; Woodward, Perkins, & Brown, 2010). Moreover, the drainage and discharge of contaminated waters lead to an increase in the quantity of exogenous chemicals spreading throughout freshwater ecosystems (Carpenter, Stanley, & Zanden, 2011; Durance & Ormerod, 2009; WWAP, 2017). Along with these direct anthropogenic impacts, climate change has been pointed out as an important driver of freshwater transformation (Carpenter et al., 2011; Michener, Blood, Bildstein, Brinson, & Gardner, 1997; Woodward et al., 2016; Woodward et al., 2010). In this way, freshwater biodiversity is nowadays widely recognised as being severely threatened as a direct or indirect result of the human footprint. Thus, it urges to establish and maintain the effort towards monitoring freshwater biodiversity loss, unravelling the mechanisms involved and implementing adequate mitigation and remediation measures.

The biota responds to environmental changes through different physiological and ultimately evolutive mechanisms provided that escaping (through e.g. migration or dispersal) from the environmental insult is prevented, which is a common scenario in highly fragmented freshwater ecosystems (Dudgeon et al., 2006; Woodward et al., 2010). Whilst phenotypic plasticity broadens physiological tolerance ranges under fluctuating environmental conditions, it rarely protects the population from the loss of fitness when the environmental challenge persists in the long-term (Gienapp, Teplitsky, Alho, Mills, & Merilä, 2008; Merilä & Hendry, 2014). In such scenarios, resistance broadening is more likely achieved through genetic adaptation configuring consistent microevolutive patterns (Karell, Ahola, Karstinen, Valkama, & Brommer, 2011; Merilä & Hoffmann, 2016). Epigenetic changes, i.e. chemical changes in the genome other than those in the DNA sequence, have been argued to play as drivers of both mechanisms of biological response to environmental challenges (Burggren, 2016; Keller, Lasky, & Yi, 2016; Kronholm & Collins, 2016). On the one hand, epigenetic modifications can mediate phenotypic plasticity ranges in critical traits of freshwater organisms responding to environmental stressors, by regulating gene expression (Baerwald et al., 2016). On the other hand, epigenetic modifications (especially those involving DNA methylation) may not be reset between generations, which can result in a transgenerational stability of these marks. In this context, environmentally induced epialleles can enrich the ground for natural selection to act, similarly to alleles at genetic loci, thus acting as drivers of genetic adaptation or steps in an adaptive walk (Kronholm & Collins, 2016; Shea, Pen, & Uller, 2011; Verhoeven & Preite, 2014).

Feeding from the above conceptual scaffold, which is essentially summarised in Figure 1, the current Synthesis focuses specifically on the putative role of the epigenome in the resilience of freshwater species to the environmental transformation induced by climate change. Although these strings on the responses of the freshwater biota to climate change and the ecological and evolutionary relevance of the epigenome have been holding per se fruitful discussions (see Woodward et al., 2010 and Verhoeven, VonHoldt & Sork, 2016 as respective examples), their melting towards depicting the existent integrated knowledge already produced and the extraction of future meaningful research

avenues is yet to be done. As a primary contribute to this integrative effort, here we first present a global picture of the most significant drivers of freshwater transformation. The impacts of climate change-related stressors on freshwater ecosystems are then scrutinized and, in this context, the evolutionary role of epigenetic mechanisms is addressed by focusing on epigenetically determined phenotypes and their inheritance across several generations. Theoretical considerations and key experimental findings are presented, and an integrative synthesis on these topics is made. Focusing on freshwater biota, future research avenues are highlighted, and the key molecular targets to be tackled, as well as the most suitable freshwater model species for the purpose will be discussed.

Revisiting the Essential Climate Change Stressors Affecting Freshwater Ecosystems

Climate change has been happening at an unprecedented pace and projections suggest that it will further cause profound alterations in freshwater systems within a few years (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Woodward et al., 2010), whereas they are already pressured by a myriad of anthropic stressors and heavily exploited for goods and services (Carpenter et al., 2011; Foley, 2005; Karr & Dudley, 1981). In this way, the claim by Woodward et al. (2010) that "*freshwater biodiversity is disproportionately at risk on a global scale"* can be appropriately quoted. Climate change is transforming the composition, biodiversity and functioning of numerous freshwater ecosystems (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Williams, 1987; Woodward et al., 2016; Woodward et al., 2010), as examined and revised by several authors (see Table 1 for a synthesis of their major conclusions). Warmer temperatures, greater variability of precipitation and higher water salinities have been highlighted as the most important derived stressors for freshwater ecosystems (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Dudgeon et al., 2006; Kefford et al., 2016).

Warmer temperatures lead to an increased melting of ice sheets and permafrost degradation (Alley, 2000), as well as to changes in evaporation and precipitation ratios (Cañedo-Argüelles et al., 2013; Carpenter et al., 2011). These are direct sources of physic limnological changes, provoking variations in the seasonality and magnitude of hydrologic income (Fenoglio, Bo, Cucco, Mercalli, & Malacarne, 2010; Hering et al., 2010). Warmer water temperatures decrease the amount of dissolved

oxygen (Hering et al., 2010; Jackson et al., 2001) and promote the decrease of habitat ranges of several aquatic organisms through their life cycle, the most iconic example being that of salmonids (Hoegh-Guldberg et al., 2007; Mohseni, Stefan, & Eaton, 2003; Schindler, 2001).

Mainly provoked by sea level rising, the salinization of freshwater ecosystems should have severe impacts on freshwater biodiversity, as it affects the survival and reproduction of several species which are typically poorly tolerant to variations in salts concentration (Carpenter et al., 2011; Gonçalves, Castro, Pardal, & Gonçalves, 2007; Martínez-Jerónimo & Martínez-Jerónimo, 2007; Mimura, 2013; Woodward et al., 2010). Even relatively small changes in salinity have been argued or shown to have an impact on freshwater and brackish ecosystems by depleting biodiversity and changing their dynamics and functioning (Cañedo-Argüelles et al., 2013; Kefford et al., 2016; Loureiro, Pereira, Pedrosa, Gonçalves, & Castro, 2013).

Besides the steady climate change trends, it is important to detail in addition that higher unpredictability in derived stressors is expected over time (Clarke, 2009; Woodward et al., 2016). Such unpredictability translates into an increased scope and frequency of environmental fluctuations, which constitutes overall a marked challenge to freshwater biodiversity (Jackson, Loewen, Vinebrooke, & Chimimba, 2016; Woodward et al., 2016).

Adaptive Strategies to Climate Change-Related Stressors

Once the salinity levels and/or temperature increases in a freshwater ecosystem, organisms can only survive and reproduce if they adapt themselves to the new environment or if they behaviourally can avoid the stressor (Berg et al., 2010; Kefford et al., 2016; Nielsen, Brock, Rees, & Baldwin, 2003) most straightforwardly by escaping into more suitable habitats (Wong & Candolin, 2015) (see Figure 1). In fact, behavioural traits such as avoidance/escaping mechanisms have been recognised as early-warning tools for signalling environmental perturbation (e.g. Peterson et al., 2017); there has also been some questioning on the most commonly applied methods to assess the biological and ecological

effects of stressor exposure since these methods reflect spatially-limited scenarios where organisms are forcedly exposed, which necessarily impairs the ecological relevance of the outcome (e.g. Araújo, Moreira-Santos, & Ribeiro, 2016).

Lentic and semi-lotic freshwater ecosystems are generally isolated and physically fragmented within large terrestrial landscapes (Dudgeon et al., 2006; Woodward et al., 2010). Thus, freshwater species inhabiting these freshwater ecosystems cannot easily migrate, disperse into a new habitat or avoid the stressors while the environment is changing (Woodward et al., 2016; Woodward et al., 2010). Because of this decreased likelihood of a successful escape, freshwater species must adapt or will perish when facing the environmental fluctuations induced by climate change (Dudgeon et al., 2006; Eros & Grant, 2015; Woodward et al., 2010). Therefore, studying the adaptive potential of freshwater species is essential to understand the effects of climate change in the biota and consequently on ecosystems structure and function (Heino, Virkkala, & Toivonen, 2009; Loureiro, Castro, Cuco, Pedrosa, & Gonçalves, 2013; Woodward et al., 2016; Woodward et al., 2010).

In this context, adaptive strategies relying on phenotypic plasticity or genetic changes assume particular relevance in their response to environmental challenges (Boutin & Lane, 2014; Merilä & Hendry, 2014; Wong & Candolin, 2015). Unlike avoidance/escaping movements, both phenotypic plasticity and microevolution by genetic adaptation are important mechanisms in the prevention of local extinction by driving population resilience (see, generally, Figure 1). In the context of climate change, phenotypic plasticity assumes particular relevance as this process potentially allows organisms to cope with the unpredictability of environmental stressors over time (Bräutigam et al., 2013). In addition, the extension of phenotypic plasticity traits across generations may have an important role in the long-term, allowing species to fine-tune their responses to climate change-related stressors (Burggren, 2016; Rey, Danchin, Mirouze, Loot, & Blanchet, 2016).

A wide diversity of organisms is known to produce a broad range of phenotypes to cope with environmental transformations (Crozier & Hutchings, 2014; Pigliucci, 2005; Vannier, Mony, Bittebière, & Vandenkoornhuyse, 2015). Such multiple phenotypes arising from a single genotype allow a broader tolerance of organisms to environmental changes and the acclimation to environmental fluctuations typical of climate change scenarios (Hoffmann & Sgrò, 2011; Merilä & Hendry, 2014; Pigliucci, 2005). However, when a given environmental stress is sustained in the long-term, phenotypic plasticity can rarely support a successful response, i.e. mitigate the loss of fitness (Gienapp et al., 2008; Merilä & Hendry, 2014). Due to the fast pace of environmental transformation provoked by climate change, it has been argued that microevolution is the key process in the long-term adaptation of species to the phenomena (Karell et al., 2011; Merilä & Hoffmann, 2016), being consequently critical to prevent extinction events. Nevertheless, far more accurately designed empirical studies are required to improve the current knowledge on the evolutionary trends under stress scenarios promoted by climate change (Gienapp et al., 2008; Merilä & Hendry, 2014). Importantly, while genetic change can indeed extend the adjusting capacity of populations facing environmental fluctuation as allowed by phenotypic plasticity (see Merilä & Hendry, 2014 for a comprehensive review on the interplay of these two processes, and Scheiner, 2014 and Scheiner, Barfield, & Holt, 2017 for key aspects regarding genetic assimilation), selection of better fit phenotypes can be costly, for example by reducing intra-population genetic variability through genetic erosion (e.g. Fasola, Ribeiro, & Lopes, 2015; Ribeiro & Lopes, 2013) or by trading-off with decreased tolerance to new stressors (Janssens, Dinh Van, Debecker, Bervoets, & Stoks, 2014; Kelly, DeBiasse, Villela, Roberts, & Cecola, 2016; Venâncio, Ribeiro, Soares, & Lopes, 2018). In this context, genetic changes are normally understood as those involving the alteration of gene sequences, i.e. the alteration of frequencies of different alleles. However, epigenetic mechanisms have been argued to play an additional role in microevolution under challenging environmental scenarios.

Epigenetic Mechanisms and their Evolutionary Role

Epigenetic mechanisms consist of potentially heritable changes in gene activity, function or expression without altering the underlying DNA sequence (Bernstein, Meissner, & Lander, 2007; Bird, 2007; Goldberg, Allis, & Bernstein, 2007; Jaenisch & Bird, 2003). Epigenetic mechanisms include (i) the chemical modification of DNA bases through the addition of methyl group (DNA methylation); (ii) the modification of histones associated with DNA (protein modifications) as they respond to environmental changes constraining chromatin structure and DNA accessibility (Bird, 2002, 2007; Goldberg et al., 2007; Kouzarides, 2007); (iii) non-coding RNAs that can control gene expression, forming complex RNA regulatory networks of the genome (Klimenko, 2017; Mattick & Makunin, 2006; Rinn & Chang, 2012). A comprehensive understanding on whether and how epigenetic mechanisms can be induced by environmental changes, as well as on their relative importance as a directed strategy for coping with environmental changes compared to stochastic epimutations, are crucial for interpreting the genomes under the influence of physiological factors (Baccarelli & Bollati, 2009; Feil & Fraga, 2012; Ho & Burggren, 2010; Leung, Breton, & Angers, 2016).

Epigenetics has been increasingly gaining relevance in several fields, mainly due to studies revealing links between epigenetic mechanisms and several key molecular and cellular processes, such as transcriptional silencing, chromosome inactivation, transposable element regulation, development and tumorigenesis (Hu & Baeg, 2017; Srinageshwar, Maiti, Dunbar, & Rossignol, 2016; Weinberg & Morris, 2016). One such field is evolutionary biology (Burggren, 2016; Laland et al., 2015; Verhoeven et al., 2016) since while it is commonly assumed that the molecular basis of the evolutionary processes are random mutations followed by natural selection (Avise & Ayala, 2009; Jablonka & Lamb, 2007), these processes fail to explain several evolutionary phenomena (Burggren, 2016; Vogt, 2017; Laland et al., 2015). For example, they fail to explain some cases of rapid adaptation commonly observed in natural populations as strictly based on the progressive accumulation of small genetic and phenotypic differences (Bernardi & Bernardi, 1986).

Numerous environmental factors have been shown to influence epigenetic mechanisms (Blake & Watson, 2016; Guillette, Parrott, Nilsson, Haque, & Skinner, 2016; Willbanks et al., 2016). As epigenetic mechanisms can regulate gene expression, the consequence of a given environmental stress scenario can be a change in the phenotype (Burggren, 2016; Manjrekar, 2017; Moore, Le, & Fan, 2013). Moreover, epigenetic alterations are generally cleared and re-established at each generation, but some of them can be inherited through successive generations even when the initial stress pressure is alleviated (Manjrekar, 2017; Daxinger & Whitelaw, 2010; Vandegehuchte & Janssen, 2014). By this means, environmentally induced epigenetic modifications and their resulting phenotypes can be inherited through successive generations (Bräutigam et al., 2013; Burggren, 2016; Anway, Cupp, Uzumcu, & Skinner, 2005). Burggren (2016) highlighted that epigenetically inherited phenotypes can be understood in the same sense as genetically inherited phenotypes, since they can be neutral, advantageous or disadvantageous for the organisms from the natural selection perspective. In this way, it is fair to assume that epigenetically inherited phenotypes can impact the overall fitness of the organisms that carry such traits and if advantageous, they can increase their adaptive capacity (Bossdorf, Richards, & Pigliucci, 2008; Burggren, 2016; Verhoeven et al., 2016; Varriale, 2014). Despite this similarity, there could be significant differences between epigenetically determined phenotypes and genetically determined phenotypes, the most functionally dramatic being the fact that, when environmental fluctuations are into place, epigenetically determined phenotypes arise more rapidly and far more broadly than genetically determined phenotypes (Burggren, 2016; Ho & Burggren, 2010; Jablonka & Lamb, 2007; Manjrekar, 2017; Vogt, 2017). Further supporting this reasoning, it is well established that DNA methylation is an important facilitator of genome mutation and it has been shown that the rates of base mutation are remarkable higher in methylated cytosines than in unmethylated cytosines (Glastad, Goodisman, Yi, & Hunt, 2016; Jiang et al., 2014; Denissenko, Chen, Tang, & Pfeifer, 1997; Nabel, Manning, & Kohli, 2012). Interestingly, Qu et al. (2012) showed that CpG methylation is a major determinant of proximal natural genetic variation. Whether the extent of the increase in mutation rates of methylated cytosines compared to non-methylated cytosines is enough to promote an evolutionary response to fluctuating environmental scenarios is still unclear, but its significant role in the responses to long-term pressure following

global climate change trends is reasonable to assume. Still, epigenetic variation can be additionally generated by epigenetic drift (errors accumulated during mitotic divisions), epimutations and epigenetic polymorphisms (Keller et al., 2016; Vogt, 2017; Leung et al., 2016). Therefore, it urges to unveil the potential relationships between these different epigenetic phenomena as it has been suggested that they can act together towards promoting a better response to environmental variations (Leung et al., 2016). This may potentially allow a less strict interpretation of the value of increased methylation rates of methylated cytosines under fluctuating environments reflecting climate change.

Most individuals within a given natural population usually experience exposure to an environmental stressor at the same time and in the same extent (Burggren, 2016). When a group of organisms is exposed to a certain stressor, the same epigenetically determined phenotypes are known to be consistently acquired (Feil & Fraga, 2012; Klironomos, Berg, & Collins, 2013; Manjrekar, 2017), confirming that a given environmental stressor can thrive into the same epigenetic modifications (and their resulting phenotypes) in the different exposed organisms (Burggren, 2016; Weyrich et al., 2016). Furthermore, the analysis of DNA methylation in wide-ranging taxa revealed that the patterns of DNA methylation are conserved across deep phylogenies (Mendizabal, Keller, Zeng, & Yi, 2014; Sarda, Zeng, Hunt, & Yi, 2012; Suzuki & Bird, 2008); and the genomic regions that reflect divergence of DNA methylation between related species seem to be enriched for both tissue and development specializations (Hernando-Herraez et al., 2013; Mendizabal et al., 2014; Wang, Cao, Zhang, & Su, 2012).

Currently, the evolutionary role of epigenetic mechanisms is a major and popular topic of scientific discussion. Several authors have been embracing the idea that the framework of the Modern Synthesis needs to be extended, namely by incorporating missing pieces in the arenas of developmental bias, phenotypic plasticity, niche construction and extra-genetic inheritance (Jablonka & Lamb, 2007; Laland et al., 2014; Pigliucci & Muller, 2010). In fact, it has been suggested that epigenetic mechanisms are important to the speciation process by enlarging the range of phenotypes available for the action of Natural Selection and further increasing of the speed of speciation (Laland

et al., 2014; Mendizabal et al., 2014; Schrey, Richards, Meller, Sollars, & Ruden, 2012). Other specific fields linking evolutionary biology to the natural dynamics of species, namely ecology, ecotoxicology and conservation biology, can also benefit from a better understanding of epigenetic mechanisms (Allendorf, Hohenlohe, & Luikart, 2010; Bossdorf et al., 2008; Mendizabal et al., 2014; Vandegehuchte & Janssen, 2014). As an illustration, the study by Vogt et al. (2015) can be cited as one stressing the potential importance of epigenetic mechanisms in the establishment of the freshwater marbled crayfish as a new species (*Procambarus fallax* f. *virginalis*), but epigenetic modifications are also important as triggers of rapid phenotypic plasticity increase, helping invasive species to thrive in different environments (Ardura, Zaiko, Morán, Planes, & Garcia-Vazquez, 2017; Chown et al., 2015; Pu & Zhan, 2017). Climate change is fostering the expansion of invasive species worldwide, which in turn are being recognised as one of the most dangerous threats to freshwater biodiversity and ecosystems (Table 1; Allen, Smith, & Darwall, 2012; Fenoglio et al., 2010; Friberg, 2014). It is indeed likely that by better exploring the role of epigenetic mechanisms enabling the expansion of invasive species in freshwater habitats, the development of better and more efficient management strategies gains additional support.

The summarized findings concerning epigenetically modified phenotypes and the transmission of epigenetic marks across generations support new perspectives shedding new light in the study of species adaptation to climate change. In this context, studies exploring the link between epigenetic inheritance, phenotype determination and natural selection are those more immediately required in order to clarify the evolutionary role of epigenetic mechanisms. Empirical data deriving from such studies are indeed crucial to build feasible new theoretical considerations in the field of evolutionary biology.

Epigenetic Contribution for the Adaptation to Climate Change

In a climate change context, extreme climatic events, the exacerbation of existing pollution and alien species invasion, rising of (water) temperatures, greater variability in precipitation patterns and higher levels of salinity have been set forward as the most important threats to freshwater biodiversity and

ecosystems (Bush & Hoskins, 2017; Cañedo-Argüelles et al., 2013; Carpenter et al., 2011; Jackson et al. 2016; Markovic, Carrizo, Kärcher, Walz, & David, 2017; Woodward et al., 2016; Woodward et al., 2010). Focusing mostly on temperature and salt stress, several studies explored the role of epigenetic mechanisms in the short-term responses of different species using various techniques and examining different epigenetic mechanisms, as synthesised in Table 2.

For example, Kumar and Wigge (2010) showed that the short-term adaptation of *Arabidopsis thaliana* to temperature changes is partly mediated through histone modification. Later, Suter and Widmer (2013) found that the exposure of several generations of *A. thaliana* to increased salt concentrations and heat conditions induced heritable phenotypic changes, but interestingly, the heritable effects of the heat exposure disappeared in the second non-exposed generation. Globally, these findings reveal that climate change-related stressors can induce heritable phenotypic changes that are epigenetically determined. The same research avenue was explored by Weyrich et al. (2016), investigating the response of wild guinea pigs (*Cavia aperea*) to rising temperatures. The authors exposed adult male guinea pigs (F0) to an increased ambient temperature and then compared the liver (as the main thermoregulatory organ) of the F0 fathers and F1 progeny, finding "*an 'immediate' and 'heritable' epigenetic response"* (Weyrich et al., 2016). More precisely, differentially methylated patterns were noticed in the exposed F0 organisms and transferred to the subsequent F1 generation. These and other epigenetic modifications are supposed to have an important ecological relevance, justifying why wild guinea pigs can thrive in habitats with remarkable differences in altitudes and temperatures ranges. Accordingly, epigenetically determined phenotypes have been proposed to be crucial in clonal species, allowing them to live in a wide range of habitats and geographical regions, despite their genetic uniformity (Leung et al., 2016; Vogt, 2017).The exposure of a parthenogenetic population of *Artemia* to a non-lethal heat shock resulted in an increase in the levels of the heat shock protein 70, which configured increased tolerance to heat stress and additional resistance against the pathogenic bacteria *Vibrio campbellii* (Norouzitallab et al., 2014); the acquired phenotypic traits were transmitted to three successive non-exposed generations and correlated with altered levels of global DNA methylation and acetylated histones H3 and H4. However, these environmentally driven

epigenetic modifications were not confirmed in *Artemia franciscana* exposed to Cd and Zn under increased temperature conditions (Pestana et al., 2016). On the other hand, Asselman et al. (2015) studied global cytosine methylation patterns following exposure of two different *Daphnia magna* genotypes to 15 stressor gradients and found that salinity was a single abiotic factor significantly affecting global DNA methylation levels in both clones tested. Indeed, DNA methylation levels in *Daphnia* (a key species in freshwater ecosystems, widely considered among regulatory frameworks) are directly influenced by the environment, and the organisms are known to produce specific epigenetic phenotypes according to the stressors in the environment (Burggren, 2016). These epigenetic phenotypes can be inherited in successive generations of daphnids, even after stressors have been removed (e.g. Asselman et al., 2017; Andrewartha & Burggren, 2012). These studies then support arguments on the decisive role that epigenetic mechanisms may have in constraining the response of freshwater populations to environmental changes

Box 1. Techniques used to assess the contribution of epigenetic modifications and patterns in Ecology and Evolutionary fields.

The study of DNA methylation, i.e. the chemical modification of the genome involving the covalent addition of a methyl group generally at cytosine residues within CpG dinucleotides, is the most common approach to address epigenetic mechanisms and the techniques available for the purpose, as well as their advantages and disadvantages were comprehensively reviewed by Kurdyukov & Bullock (2016). This study largely supports our highlights below regarding DNA methylation analysis, although specific references are quoted for uncovered techniques or considering the particular scope of the present Synthesis. As follows, DNA methylation can be measured globally, which is the most frequent record, or relying in sequencing approaches.

- **Global DNA methylation levels** are most commonly assessed based on **High Performance Liquid Chromatography (HPLC)** coupled with **UV spectrophotometry** or **mass spectrometry** (e.g. LC-MS). In these techniques and following DNA digestion, single nucleotides are separated according to their size for further quantification of both cytosine and methylated cytosine; the relative percentage of 5-methylC compared

to total cytosine pictures global methylation levels. Although these are highly reproducible and feasible techniques, they require large amounts of DNA. In addition, they cannot give any single base information or gene level information and only report changes in global or total methylation levels. Hence alternatives have been developed that generally include PCR-based methods and can be more adequate for specific applications.

Sequencing approaches are becoming more common as the analysis of gene-specific epigenetic changes could increase the quantity and specificity of the gathered information, allowing a dramatically better understanding of the biological role of DNA methylation. In addition, this strategy makes it easier to find correlations between methylation status and phenotype traits. Among sequencing approaches the following have been gaining increased attention.

- **Bisulfite sequencing** is a DNA sequencing approach in which, prior to sequencing, DNA undergoes a bisulfite treatment where unmethylated cytosines are converted to uracil and methylated cytosines are unaffected. Specific protocols such as MethylCSeq exist supporting the technique (Urich, Nery, Lister, Schmitz, & Ecker, 2015).
- **Reduced representation bisulfite sequencing** (RRBS) is an efficient and cost-effective variation of bisulfite sequencing commonly used to analyse gene-specific methylation levels (Meissner et al., 2005). Prior to the bisulfite treatment, DNA undergoes an enzymatic digestion using a methylation-insensitive restriction enzyme to enrich for CpGs. By doing so, the cost of sequencing will decrease as only the enriched regions of the genome will be sequenced.
- Methyl Binding Domain Sequencing (MBD-Seq) uses a methyl binding domain (MBD) to capture methylated DNA fragments (Lan et al., 2011). Only methylated DNA fragments captured with the MBD are used for library preparation and sequencing.
- **Methylated DNA immunoprecipitation** (MeDIP) is based on the enrichment of methylated DNA using monoclonal antibodies that specifically recognise 5-methylcytidines and/or 5-methylcytosines. DNA must be first denatured into single stranded molecules for further immunoprecipitation. The purified methylated DNA can then be used for locus-specific (via PCR) or genome-wide (using e.g. sequencing platforms) methylation studies. MeDI provides high-level enrichment of genomic regions with low CpG density and does not requires high-quality DNA, but it has been shown to perform inferiorly compared to restriction-based methods such as RRBS.
- **Methylation-sensitive amplified polymorphisms** (MSAP) is a modification of amplification fragment length

polymorphism (AFLP) analysis that is also efficient to profile methylation patterns without requiring detailed knowledge of the genome sequence. The technique is based on DNA restrictive digestion with EcoRI /recognizes the GAATTC site) combined with each of two isoschizomeric endonucleases (*Msp*I and *Hpa*II) bearing differential sensitivity to cytosine methylation, followed by the amplification of restriction fragments and separation in denaturing polyacrylamide gels (Fulneček & Kovařík, 2014).

The study of histone modifications and non-coding RNAs is still very limited, possibly because more fundamental research is still required regarding both these mechanisms. Nevertheless, the few studies that can be mentioned in the context of our Synthesis are highlighted in Table 2. Among these works, high performance liquid chromatography and chromatin immunoprecipitation have been used to perform the job (e.g. Kumar & Wigge, 2010; Norouzitallab et al., 2014) and their principles are as follows.

- **High performance liquid chromatography (HPLC)** separates protein molecules by molecular weight and conformation. The method is usually combined with mass spectrometry (MS) (Minshull, Cole, Dockrell, Read, & Dickman, 2016). After the physical separation of histones by HPLC, MS allows the detection of histone post-translational modifications.
- **Chromatin immunopurification (ChIP)** involves the purification of nucleosomes after chromatin fragmentation with MNase for further immunoprecipitation of modified nucleosomes (Park, 2009). Relative enrichment of modified histones with associated DNA fragments is normally analysed by qPCR.

Despite the interesting results obtained so far, the role of epigenetic mechanisms in constraining biological and ecological responses in the long-term is particularly relevant since these are those cues allowing improved rationales in evolutionary arenas. Nevertheless, the literature is scarce on this topic possibly because such experiments require the monitoring of multiple generations and hence demand long time and effort. Therefore, the few references that can be highlighted regarding aquatic systems have only documented the epigenetic response to environmental perturbations in a single or at most two generations. Moreover, population level studies are also scarce (but see below) although critical to fully understand the evolutionary role of epigenetic mechanisms.

Epigenetic variation has been touted to be an important driver of evolution, but a better understanding of transgenerational inheritance of adaptive epigenetic patterns is still required, as well as the collection of better insights on the selection of epigenetically determined phenotypes and their genetic fixation in the long-term (Vogt, 2017; Kronholm et al., 2017). Under this rationale, Kronholm et al. (2017) manipulated DNA methylation and histone acetylation in the unicellular green alga *Chlamydomonas reinhardtii* both genetically and chemically. The authors could monitor the amount of epigenetic variation generated or transmitted in populations adapting to three different challenging environments (salt stress, phosphate depletion, and high CO_2 levels) followed for two hundred asexual generations. They observed that, by reducing the amount of epigenetic variation available in the populations, adaptation to the different stressing environments is limited while the opposite would happen when levels of epigenetic variation were kept unchanged. These results highlight that transgenerational epigenetic effects indeed have an important role in adaptive evolution and suggest that different methylation changes can result in distinct evolutionary outcomes.

Another important piece of the puzzle was presented by Varriale (2014) while reviewing on the evolutionary role of epigenetic mechanisms in vertebrates and focusing on previous records regarding the variation of global DNA methylation levels of several cold- and warm-blooded vertebrates (e.g. Jabbari, Cacciò, Païs De Barros, Desgrès, & Bernardi, 1997; Varriale & Bernardi, 2006a, 2006b). Since the levels of methylation of tested warm-blooded species were consistently lower than those of the cold-blooded vertebrates, it was suggested that the environment directly influenced the methylation composition of genomes over evolutionary time scales, and then the claim was supported by showing that regardless phylogenetic distances (fish inhabiting polar, temperate and tropical regions), there was a significant negative correlation between methylation and temperature (Varriale, 2014). In summary, this particular example highlights that different methylation compositions of the genome can directly result in different phenotypes being produced and selected over evolutionary time scales. While the previous findings helped to unveil the circumstances and mechanisms leading to the arise of epigenetically determined phenotypes in vertebrates, their maintenance and stable transmission over evolutionary times remains largely unexplored among

freshwater ecosystems. An exception is the study of Artemov et al. (2017), who investigated the role of DNA methylation in the adaptation of populations of the marine stickleback (*Gasterosteus aculeatus*) to freshwater conditions. Notably, the DNA methylation profile of marine sticklebacks transferred into freshwater partially converged to that of a freshwater stickleback, with the genes encoding ion channels (KCND3, CACNA1FB, and ATP4A) being differentially methylated between the marine and the freshwater populations. Even more important, the authors noticed that the short-time exposure of marine organisms to freshwater conditions provoked specific DNA methylation changes that corresponded to the methylation patterns of freshwater populations. Therefore, it seems that an immediate epigenetic response to freshwater can indeed be maintained in the long-term. In agreement to these results, the potential importance of epigenetic mechanisms in constraining the evolution of K-strategist species was stressed by Lighten et al. (2016), who compared the transcriptome of two recently diverged populations of the winter skate (*Leucoraja ocellata*) and found that epigenetic mechanisms determined the different profiles of gene expression observed in the populations; in addition, a relevant portion of the differentially expressed transcripts was correlated to genes whose function is involved in the different life-history traits of the populations.

These studies corroborate that epigenetic mechanisms indeed have an important evolutionary role. More precisely, some of these studies provide *in vivo* evidence that epigenetic mechanisms are capable of shaping the genome of organisms, allowing long-term adaptations of populations to environmental changes (Bernardi & Bernardi, 1986; Jablonka & Lamb, 2007; Wang, Crutchley, & Dostie, 2011). Particularly in a climate change scenario, there is strong evidence that epigenetic mechanisms contribute to the widening of phenotypic plasticity ranges, and consequently to the adaptive capacity of several organisms, including plants and fishes (Bräutigam et al., 2013; Munday, 2014; Rey et al., 2016; Vannier et al., 2015). Focusing on climate change, Rey et al. (2016) proposed a molecular engine that combines epigenetic mechanisms and transposable elements to explain how organisms can adjust their phenotypes, regulate the production of phenotypic and genetic variation, and stably transmit the phenotypes across generations.

Conclusions and perspectives for future research

It is becoming increasingly evident that epigenetic mechanisms are indeed involved in the evolutionary adaptation of species to climate change. However, the adaptive potential of freshwater populations to climate change is still poorly understood, and both short- and long- term studies are needed to clarify the routes and extent of the contribution by epigenetic mechanisms (Kefford et al., 2016; Munday, 2014; Rey et al., 2016). More broadly, due to epigenetic transgenerational inheritance, environmental perturbations can extend their effects in the long-term triggering population adaptation, which may impact ecosystem functions (Mirbahai & Chipman, 2014; Shaw et al., 2017). Consequently, further transgenerational (see Hanson & Skinner, 2016, for context and definition of trans- vs multi-generation experiments) studies are required to better understand the role of epigenetics in constraining the adaptation of freshwater biota to climate change stressors (Bell & Stein, 2017; Shaw et al., 2017; Vandegehuchte & Janssen, 2014). Moreover, such transgenerational studies should focus not only in the epigenome of the exposed generations but also in the epigenome of subsequent non-exposed generations (Andrewartha & Burggren, 2012). The definition of a non-exposed generation can be particularly challenging in organisms showing external fertilisation and/or internal embryo development in a permeable brood chamber such as e.g. cladoceran species. In fact, in such cases the somatic and/or germline cells of developing embryos can also be directly exposed to the parental stressor depending mostly on exposure length and on the exposed life-stage (Bell & Stein, 2017; Harris et al., 2012; Shaw et al., 2017).

One possible approach to tackle these challenges more promptly (following genome-wide methylation profiling or independently provided the adequate techniques become tuned) is by focusing specifically on the molecular mechanisms and cellular pathways that regulate the susceptibility of freshwater species to temperature and salinity fluctuations. For example, and as far as salinity fluctuations are concerned, the sodium pump (Na^+/K^+-ATPase) is the main responsible for osmoregulation in freshwater crustaceans, thus the genes encoding and regulating expression of this protein group are interesting candidate targets to look at in this context (Bianchini & Wood, 2008; Henry, Lucu, Onken, & Weihrauch, 2012; Latta, Weider, Colbourne, & Pfrender, 2012; Sáez, Lozano,

& Zaldívar-Riverón, 2009). Moreover, the critical role of heat shock proteins in stress tolerance in general, and heat stress in particular, has been highlighted for several freshwater species and marine invertebrates (Matthews, 2012; Pestana et al., 2016; Solan & Whiteley, 2016). Thus, this set of genes and their regulators also seem promising candidates for future research regarding adaptation to climate change. In addition, examination of gene-specific methylation status would enhance the detection of transgenerational epigenetic effects, which are more informative regarding adaptation strategies and the heritability driving evolution (Harris et al., 2012; Vandegehuchte, De Coninck, Vandenbrouck, De Coen, & Janssen, 2010). Currently the tools for the job are available, and techniques such as bisulphite sequencing, methylated DNA immunoprecipitation (meDip) or DNA methylation sensitive restriction enzyme digests allow gene-specific analyses (Harris et al., 2012; Kurdyukov & Bullock, 2016). As far as histone modifications and non-coding RNAs are concerned, more fundamental research is still required to foster the clarification of the potential role of both epigenetic mechanisms in the response of organisms to environmental change (e.g. climate change scenarios).

Finally, the availability of well-known models for addressing epigenetics in the freshwater biota is pivotal to boost a better understanding of the responses to climate change by these ecosystems. Regarding freshwater communities, *Daphnia* has been touted as an epigenetic model (Bell & Stein, 2017; Brander, Biales, & Connon, 2017; Harris et al., 2012). The major underlying reason for this statement is the reproductive strategy of these animals, which allows the establishment of parthenogenetic clones with the consequent possibility of eliminating genetic variability in experimental trials (Harris et al., 2012; Weider & Hebert, 1987). Furthermore, the draft genome sequence of *D. magna* is now available, as well as a large quantity of ecological, ecotoxicological and evolutionary literature on *Daphnia* species that can facilitate the understanding of gene-environment interactions (Baudo, 1987; Bell & Stein, 2017; Harris et al., 2012). Besides *Daphnia*, the marbled crayfish has been suggested as a suitable model organism for epigenetic, environmental epigenomics and evolutionary studies (Vogt, 2008). In fact, it has been used for experiments in different fields and bears the advantage of being a vigorous, clonable and eurytopic organism (Vogt, 2008, 2017; Vogt et

al., 2015). The zebrafish (*Danio rerio*) seems also a suitable vertebrate model for epigenetic research, particularly in development and disease studies (Detrich, Westerfield, & Zon, 2016; Martinez-Sales, García-Ximénez, & Espinós, 2015; Mudbhary & Sadler, 2011), although necessarily more representative of tropical ecosystems. This advanced position of zebrafish is unquestionably sustained by its representative use both in (eco)toxicological and biomedical research (e.g. Dai et al., 2014; Szabo et al., 2017), but its representativeness as a model in ecology and evolution can be challenged (Parichy, 2015). In this context, other fish species such as *Chrosomus eos-neogaeus* can potentially apply as suitable models for epigenetic research regarding freshwater ecosystems (Leung et al., 2016).

The studies addressed in this synthesis have been helping to unveil the potential role of epigenetic mechanisms in the adaptation of species to climate change, over different temporal scales. Focusing on freshwater biodiversity, follow-up studies could provide valuable insights into the adaptive capacity of freshwater species to climate change, and mechanisms involved in their responses to habitat transitions. Ultimately, these studies could be helpful resources for decision makers, since the incorporation of transgenerational epigenetic heritability into risk assessment procedures has been highlighted recently as a facilitator of the establishment of protective measurements against biodiversity loss (Shaw et al., 2017). As climate change is contributing to the rapid decline of freshwater ecosystems around the world, more than ever, their protection is an urgent and serious issue.

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Table 1. Summary of the predicted and/or observed effects of climate change (generally driven by the increase in water temperature unless stated otherwise) on the biological, chemical and physical features of freshwater ecosystems worldwide. The analysis was restricted to lakes and rivers taken as lentic and lotic reference systems; they were separated as needed to benefit the clarity of the presentation of climate change effects.

Reference	Assessment Region	Biological effects	Chemical and Physical effects
Mulholland et al. (1997)	South-Eastern United States and Gulf Coast of Mexico	*Lakes* • Reduction of the macrozooplankton community. • Invasion of non-native species, temperate fish replaced by exotic and subtropical species. • Higher habitat constriction, particularly in summer. • Increased susceptibility to parasites and pathogens^{+}. *Rivers and Streams* • Simplified biological communities; favoring of species with short and multivoltine life histories. • Less habitat for cold water fish and macroinvertebrate species. • Changes in the distribution of aquatic insects. • Local elimination of some species, particularly acid-sensitive. • Increased loss of organisms by downstream drift during more intense flushing events. • Expansion of subtropical species northwards.	*Lakes* • Increased organic matter decomposition. • Greater runoff from urban and agricultural areas. • Increased primary production, organic matter decomposition and nutrient cycling. • Oxygen squeeze^{+}. *Rivers and streams* • Longer periods of low flow conditions, especially during the summer. • Increased episodes of stream acidification. • Changes in the salinity regimes.
Ryan and Ryan (2006)	New Zealand	• Increased dominance of filamentous green algae over diatoms and cyanobacteria in periphyton communities.	• Increased frequency of extreme climatic events, such as floods and drought.

(*)		• Simplification of invertebrate communities following adaptation to changes in resources and after recovery from extreme events. • Invertebrates mature earlier and at a smaller size. • Long-range dispersal of winged adult invertebrate limited, contributing to the decrease of genetic diversity. • Changes in the composition of riparian vegetation that may or may not impair quantity and quality of leaf inputs, thus food resources. • Proliferation of parasite infective stages. • Limitation of fish migration patterns. • Flood events may remove invertebrate food sources and scour out benthos, limiting the availability of food resources for fish. It also increases turbidity, hence lowers predatory efficacy, as well as favours fish egg loss. • Increased likelihood of successful establishment of exotic aquarium escapees.	• Longer periods of low flow. • Floods resuspend sediments, increasing turbidity. • Decreased nitrogen input in tree leaves due to increased atmospheric CO_2 levels. • Oxygen depletion as water temperature increases.
Hering et al. (2010)	Europe	*Lakes* • Earlier and enhanced phytoplankton spring growth with progressive loss of diversity through the dominance of cyanophytes and/or cyanobacteria. Enhanced macrophyte growth and deeper colonization. • Earlier, higher spring growth of daphnid grazers, followed by heavy phytoplankton suppression, and then an earlier summer decline of these large-bodied species in favour of smaller species. • Decrease of littoral habitat range of cold-water fish species, as well as reproductive success; parasitic infections and egg and young's predation increase. Planktivorous cyprinid species are favoured, which heavily suppress large-bodied zooplankton contributing to promote phytoplankton growth. • Alien fishes, macrophytes or macroinvertebrates are favoured^{+}.	*Lakes* • Shortening of ice cover periods^{+}. • Earlier onset and prolongation of summer stratification. • Increased loss of water bodies following drought. • More frequent oxygen depletion events following phytoplankton growth. • Higher sulphate concentrations via sulphur oxidation and transport following drought. • Increased frequency of acidification pulses and higher dissolved organic carbon concentration. • Increased total carbon load following higher runoff levels.

	Rivers and streams • Enhanced algae and macrophyte growth. • Increased respiration rate after favouring of detritivores and decomposers. • Disfavouring or extinction of cold stenothermic invertebrates and fish. • Increase of invertebrates r-strategists benefiting from extreme flood events. • Changes in life strategies, with univoltine invertebrates with early emergence being favoured over bivoltine or semivoltine species. • Species typical of colder, highly oxygenated water are replaced by potamal species typical of warmer waters. • Reduced reproductive success and increased parasitic and predatory pressure (eggs and youngs) over salmonids. Cyprinid fish are favoured.	• Increased sea-salt deposition. *Rivers and streams* • Increased frequency of small streams/rivers changing from permanent to temporary or intermittent. • Changes in flow regimes and channel morphology due to extreme rainstorms, increased runoff and sedimentation rates. • Increased carbon and nutrient income and wider variation of carbon, phosphorus and nitrogen mineralisation rates. • Higher dissolved oxygen depletion due to raised production and decomposition. • Higher runoff of total organic carbon and consequent acidification.

Allen et al. (2012)	Indo-Burma Region (Thailand, Vietnam, Cambodia, Lao, Myanmar)	*Lakes* • Food-web alterations[+]. • Severe impacts on highly specialized species, with complex life histories, with restricted ranges/limited distribution and/or with specific habitat requirements[+]. • Skews in sex ratios of aquatic reptiles[+]. • Lower body mass and higher metabolic rates in amphibians[+]. • Increased metabolic costs for fishes[+]. • Increased invasion of alien species and transmission of fish parasites and diseases[+]. *Rivers and streams* • Increased migration to higher elevations or latitudes, particularly species that are relatively close to their upper thermal limits. • Shifts in the timing of fish migration.	*Lakes* • Increased dry-season droughts. • Lower water oxygen levels[+]. *Rivers and streams* • More extreme flow events. • Increased saltwater intrusion and erosion.
Dallas and Rivers-Moore, (2014) (*)	South Africa	• Increased algal growth and load of cyanotoxins. • Changes in aquatic biodiversity and in the composition of communities and distribution of species. • Extinction of vulnerable species. • Alteration of individual phonologies and life-history patterns. • Higher susceptibility of species with specialised habitat and/or microhabitat requirements, narrow environmental tolerances or thresholds, dependence on specific environmental triggers, dependence on interspecific interactions and poor ability to disperse or colonise a new area. • Spread of invasive and pest species. • Increase waterborne and vector-borne diseases.	• Higher frequency and intensity of extreme events (droughts and floods). • Changes in runoff patterns (flow variability, duration, timing) and in groundwater recharge rates. • Changes in channel geomorphology (including longitudinal and lateral connectivity). • Increased sedimentation and turbidity of the water. • Lower dissolved oxygen concentration. • Changes in nutrient cycles and increased organic matter decomposition. • Higher transport of dissolved contaminants, such as pesticides and pathogens.

		• Increased mobilisation of adsorbed contaminants, such as metals and phosphorus, from the riverbed. • Higher salinization.

* Focused on lotic systems;[+] Also predicted and/or observed in rivers and streams.

Table 2. Biological effects reported concerning epigenetic responses to environmental heat and salt changes.

Epigenetic Mechanisms	Reference	Reported effects
DNA methylation	Weyrich et al. (2016)	Immediate and heritable epigenetic response found when studying the adaptation of wild guinea pigs (*Cavia aperea*) to rising temperatures
	Bind et al. (2014)	Temperature (or relative humidity) levels associated with methylation on tissue factor (*F3*), intercellular adhesion molecule 1 (*ICAM-1*), toll-like receptor 2 (*TRL-2*), carnitine O-acetyltransferase (*CRAT*), interferon gamma (*IFN-γ*), inducible nitric oxide synthase (*iNOS*), and glucocorticoid receptor, *LINE-1*, and *Alu*, in elderly human samples
	Asselman et al. (2015)	Salinity significantly affected global DNA methylation levels in both clones tested of *Daphnia magna*
	Varriale (2014)	The levels of methylation of warm-blooded species were consistently lower than those of the cold-blooded vertebrates
	Garg, Chevala, Shankar and Jain (2015)	Reported that the role of DNA methylation patterns are typically associated with genes important for abiotic stress responses in three rice (*Oryza sativa*) cultivars (IR64, stress-sensitive; Nagina 22, drought-tolerant; Pokkali, salinity-tolerant)
	Artemov et al. (2017)	Genes encoding ion channels (KCND3, CACNA1FB, and ATP4A) were differentially methylated between a marine and the freshwater populations of the winter skate (*Leucoraja ocellata*)
	Sun et al. (2016)	Both females and males from the Nile tilapia (*Oreochromis niloticus*) showed an increase in methylation levels on various chromosomes after high-temperature exposure
	Lighten et al. (2016)	Epigenetic mechanisms determined the different profiles of gene expression and life-history traits of two recently diverged populations of the winter skate (*Leucoraja ocellata*)
Histone Modifications	Kumar and Wigge (2010)	Short-term adaptation of *Arabidopsis thaliana* to temperature changes was partly mediated through histone H2A.Z-containing nucleosomes
	Lämke and Bäurle (2017)	Focusing on plants, discussed the role of chromatin in stress responses and the current evidence on somatic, intergenerational, and transgenerational stress memory
	Pestana et al. (2016)	No epigenetic alterations (global DNA methylation and histone H3 and H4 acetylation) were observed in *Artemia franciscana* exposed to Cd and Zn (after a prior exposure to non-lethal heat shock)
Non-coding RNAs	Stief, Brzezinka, Lämke and Bäurle (2014)	Discussed how microRNAs regulate the heat stress memory and increase survival upon a recurring heat stress in plants. In addition, focused on transgenerational inheritance of retrotransposition

		during prolonged heat stress
Several mechanisms	Liu, Feng, Li and He (2015)	Reviewed the role of different epigenetic mechanisms in plant heat responses, namely DNA methylation, histone modifications, histone variants, ATP-dependent chromatin remodelling, histone chaperones, small RNAs and long non-coding RNAs
	Norouzitallab et al. (2014)	Increased levels of the heat shock protein 70 (which configured increased tolerance to heat stress and additional resistance against the pathogenic bacteria *Vibrio campbellii*) were observed after exposure of a parthenogenetic population of *Artemia* to a non-lethal heat shock. The phenotypic traits were transmitted to non-exposed generations and correlated with altered levels of global DNA methylation and acetylated histones H3 and H4